REMARKS/ARGUMENTS

Reconsideration is requested.

The claims are 124 to 147.

Claims 124 to 136 remain submitted.

Favorable consideration of claims 137 to 147 is requested. The NANBV antigen having the amino acid sequence from residue 1 to 120 of SEQ ID NO: 73 is presented in dependent claim 131. Claims 137 and 140 are to the use of the full NANBV capsid antigen and is within the scope of generic claim 124. Accordingly, claim 137 and 140 should be examined together with claim 124.

Claims 138 and 141 are the embodiment wherein a NANBV capsid antigen as in claim 124 is used in combination with non-structural C-100-3 antigen and should also be examined together with claim 124. The use of the capsid antigen with the C-100-3 antigen is disclosed at the Specification, clean copy filed September 7, 2006 ("Specification") page 75, fourth full paragraph. The C-100-3 antigen is disclosed, Specification page 4, last paragraph through page 5, top. The C-100-3 antigen per se was publicly known prior to the filing of the instant patent application on August 27, 1990. See, for example, Houghton/Chiron European Patent Application No. 318316,

cited by the Examiner, page 8, lines 35 to 36 and Figure 36 showing the 363 amino acid sequence starting at position 1569 and ending at position 1931.

Claims 139 and 142 are to the use of the full NANBV capsid antigen together with non-structural C-100-3 antigen and should also be examined together with claim 124.

See also the attached paper by Kuo et al., Science, 244:362-364 (1989), cited in the Specification at page 4, line 5 of the second paragraph, describing the C-100-3 NANBV assay.

It should be noted that the Wang patent, cited by the Examiner and more fully discussed hereinbelow, refers to "HCV C-100". However, the peptide sequences shown from the C-100 region in the Wang patent, which are designated therein as I, II, III, IV, V and VI, Wang, column 18, lines 31 et. seq., are each short sequences of about 40 amino acids which span Wang's preferred immunodominant region of 90 amino acids.

Wang peptide VII (aa 1889 to 1931), column 15, lines 42 to 44, is only a short sequence of 40 amino acids at the end of the 363 amino acid sequence of C-100-3 antigen. Wang does not disclose or suggest any combination of C-100-3 antigen (containing 363 amino acids, over four times as long as Wang's immunodominant

region), with a capsid antigen in any EIA.

The prior claims were rejected on Wang, United States Patent No. 5,106,726 in view of Houghton, European Patent Application No. 318216 under 35 U.S.C. 103(a) as being obvious to one of ordinary skill at the time the invention was made.

Applicants have previously filed a Declaration Under 37 CFR 1.131 swearing back of Wang. Hence, Wang is not prior art and the rejection fails.

While Wang is not prior art, we wish to further explain that Wang does not disclose or render obvious to one of ordinary skill as of the filing date of the instant patent application, effective filing date August 27, 1990, the subject matter of either the prior claims or the claims presently pending.

The Wang patent was filed on July 26, 1990 under United States Patent Application Serial No. 07/558,799, copy of United States Patent and Trademark Office file history in included in the Declaration of Joseph E. Mueth, filed herewith.

Leaving aside the Declaration Under 37 CFR 1.131, Wang could be relied upon under 35 U.S.C. 102(e) only for what it disclosed as of July 26, 1990, that is, "enabled" in regard to the capsid amino acid sequence since Houghton European Patent Application No. 318316 has no disclosure at all of the capsid.

The Examiner particularly cites to Wang Examples 17 and 18, specifically to column 44, lines 27 to 38, and also to Wang claim 1 appearing at column 47, line 53 through to column 49, line 44.

For convenience and in the interest of clarity, we shall hereafter refer to the originally filed Specification of Wang, United States Patent Application Serial No. 07/558,799 ("Wang Specification") found in the Declaration of Joseph E. Mueth.

The cited column 44, lines 27 to 38, appears in the Wang Specification at page 73, lines 11 to 21 and claim 1, page 79 (all) through to the top of page 80 (pages 79 and 80 have no line numbers).

The Wang Specification shows how these pages read as of filing on July 26, 1990. Page 73, lines 11 to 21 read:

> (c) Serial samples from three well-characterized representative HCV seroconversion panels, collected by Serologic Inc., were tested by HCV EIA formats A, C and D, as defined in Example 15 in addition to that previously tested with rDNA HCV C-100 based EIA. As shown in Table 8, both HCV EIA formats C and D were able to identify HCV antibody positive specimens in two out of three panels by four to eight weeks earlier than the rDNA HCV-100 based EIA and HCV EIA Format A. This

further demonstrates the sensitivity of the HCV EIAs which incorporate peptides derived from the HCV structural (core) protein region.

The A, C and D formats, referred to in the above passage, are described in the Wang Specification at page 70, lines 1 to 18 as follows:

EXAMPLE 15

Detection Of Antibodies To HCV By Peptide Based Enzyme-Linked Immunosorbent Assay Using Format C, Format D, Format A

The following four groups of specimens:

(a) individuals with AIDS, ARC(n=63);

(b) individuals positive for HBsAg, (n=50);

(c) individuals positive for antibodies to HBc protein, (n=22); and

(d) individuals with elevated (>100 i.u./L) alanine aminotransferase (ALT) enzyme activity, (n=86).

were analyzed on representative HCV peptide based EIAs according to the present invention, with the plates coated either with (i) peptides IIH and V at 5 and 3 ug/mL each (Format A), (ii) peptides IIH, V and VIIIE) at 5, 3 and 2 ug/mL each (Format C, containing both the HCV core and nonstructural peptides) or (iii) Peptides VIIIE and IXD at 2 and 2 ug/mL each (Format D, HCV core peptides only).

The Wang Specification as filed was replete with fatal sequence errors to which we now turn.

Attached are 24 pages of the original Wang Specification with all the changes marked, and in accordance with the Preliminary Amendment filed by Wang on December 10, 1990. These changes, which are enumerated sequentially (1 to 50), are indicated by the number written in the right margin of each page, and with an arrow pointing to the change. Several of changes have been consolidated under a single number, as they are located closely spaced on the page.

The Wang patent application as filed on July 26, 1990 did not correctly disclose the peptide sequence of either peptides VIIIE or IXD, the only structural HCV sequences shown to have been subjected to EIA testing in Examples 15 to 18. Contrary to the Examiner's apparent suggestion, there is no disclosure in Wang of an EIA based on the sequence of HCV residues 21 to 40 other than as part of sequence XIIIE. Wang has a plethora of errors with regard to the stated sequence information. Sequences VIIIE and IXD originally were wrong in most occurrences and Wang later attempted to change them (see Mueth Declaration, the Preliminary Amendment filed by Wang December 10, 1990 [Wang Paper No. 3], and the Requests for Certificate of Correction filed February 25, 1993 [Wang Paper No. 10] and August 29, 1994 [Wang Paper No. 19]). The contradictory sequence information in sequences VIIIE and IXD of the original filing causes an attempt to follow Wang's original teachings to turn into an

exercise of serendipity (which table, or text segment, should have been taken as the correct information to repeat Wang's experiments?). The HCV capsid peptides (i.e., the VIII and IX series of peptides) both have errors in them. First, for peptide IX (replacing Thr with Tyr and Tyr with Thr at two different locations of the peptide) was requested on February 25, 1993 with Certificate of Correction finally issued on September 20, 1994. We note that this correction precipitated a need for a further correction (of the location, in the specification, where the correction was to be made), of one of the corrections, the final Certificate of Correction issued on December 13, 1994. Second, there was also an error in Peptide VIII, which is the N-terminal segment of the HCV capsid, where the amino acid no. 13 of said peptide (this equals amino acid no. 14 of the capsid sequence, since Wang's peptide VIII begins with aa no. 2) was first stated as His, then corrected (per Preliminary Amendment dated December 10, 1990) to Asn. These are not conservative substitutions. Wang itself teaches (column 4, lines 28-29) that the conservative substitutions are shown in Table 6 ("These show where conservative substitutions, deletions or substitutions can be made."). The errors and attempted corrections made reflect non-conservative substitutions and as such are neither typographical errors, nor irrelevant (conservative) amino acid changes.

As is pointed out eloquently in the paper by P.M. Colman, "Effects of amino acid changes on antibody-antigen interactions", Research in Immunology, No. 1, 145:33-36 (1994), including the many references in the article (which precede July, 1990), a single amino acid change within the interface of an antibody-antigen complex can have a

profound effect on the stability of the complex, the direction of which is, even today, most difficult if not impossible to predict. The conflicted original disclosure of Wang made it impossible to ascertain the antigen epitope sequence configuration which would give the results of Example 17.

In the Remarks including in the Preliminary Amendment filed December 10, 1990, Wang's counsel made the following statements:

> Support for the amendment of pages 16, 17, 18, 19, 26, 28, 29, 35, 36, and Claim 1 can be found in Table 1, Fig. 1-1, and Table 7, Fig 11-1 as originally filed. Support for the amendment of pages 24 and 71 can be found in the specification at page 24, line 24, and Example 17. Support for the amendment on page 34 is to be found in Table 7, Fig. 11-1, Fig. 11-2, and Claim 7 as originally filed.
>
> Page 38 was amended to put in the three letter codes for the amino acid sequences. The amendment on page 49 is to correct an obvious typographical error. Support for the amendment of page 75 can be found in Table 9.

It is essential to note that as of July 26, 1990, the sequence of the capsid portion of the HCV genome including HCV sequences VIIIE and IXD were not publicly known. Consequently, by presenting alternative peptide sequences for both VIIIE and IXD, Wang had nothing to point to for establishing which of the alternatives were the correct ones. Wang had no acceptable basis upon which to choose the sequences of Table 7 over the alternative sequences scattered throughout the Wang Specification (or vice versa).

The July 26, 1990 Wang patent application filing was fatally defective under 35 U.S.C. 112 by virtue of its presentation of alternative structures for the VIIIE and IXD sequences.

The Federal Circuit has held that where the invention resides is the sequence, disclosure of the sequence structure is essential to compliance with 35 U.S.C. 112.

In Fiers v. Sugano, 25 USPQ2d 1601 (Fed. Cir. 1993), the interference subject matter was DNA which codes for human fibroblast beta-interferon (β-IF), a protein that promotes viral resistance in human tissue. It involved a single count which read:

> A DNA which consists essentially of a DNA which codes for a human fibroblast interferon-beta polypeptide.

Sugano's application disclosed the complete nucleotide sequence of a DNA coding for β-IF and a method for isolating that DNA. The Fiers court held that Fiers' application did not contain a written description of a DNA coding for β-IF since it did not disclose the nucleotide sequence or "an intact complete gene." The Board, in denying Fiers' request for reconsideration, rejected the argument that it is only necessary to show some correspondence between the language in the count and language in the Israeli application to satisfy the written description requirement.

In Fiers, the court concluded:

> An adequate written description of a DNA requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it; what is required is a description of the DNA itself. Revel's [Fiers'] specification does not do that. Revel's application does not even demonstrate that the disclosed method actually leads to the DNA, and thus that he had possession of the invention, since it only discloses a clone that might be used to obtain mRNA coding for β-IF. A bare reference to a DNA with a statement that it can be obtained by reverse transcription is not a description; it does not indicate that Revel was in possession of the DNA.

Regents of the Univ. of Cal. v. Eli Lilly & Co. 119 F.3d 1559, 1566, 43 USPQ2d 1398, 1404 (Fed. Cir. 1997), *cert. denied* 523 U.S. 1089 (1998). In Eli Lilly, the patent claimed "2. [a] recombinant procaryotic microorganism modified to contain a nucleotide sequence having the structure of the reverse transcript of an mRNA of a vertebrate, which mRNA encodes insulin," U.S. Patent No. 4,652,525, col. 21, lines 1-5, and "5. [a] microorganism according to claim 2 wherein the vertebrate is a human." Id. at col. 22, lines 3-4. The claimed "reverse transcript of an mRNA [also known as "cDNA"] of a vertebrate" was not described by sequencing. Instead, the patent simply named the cDNA and described the process that could be used for isolating it. The Federal Circuit held:

> The name cDNA is not itself a written description of that DNA; it conveys no distinguishing information concerning its identity. While the example provides a process for obtaining human insulin-encoding cDNA, there is no further information in the patent pertaining to that cDNA's relevant structural or physical characteristics; in other words, it thus does not describe human insulin cDNA. Describing a method of preparing a cDNA or even describing the protein that the cDNA encodes, as the example does, does not necessarily describe the cDNA itself. No sequence information indicating which nucleotides constitute human cDNA appears in the patent, as appears for rat cDNA in Example 5 of the patent. Accordingly, the specification does not provide a written description of the

invention of claim 5.

Eli Lilly, 119 F.3d at 1567, 43 USPQ2d at 1405.

In Eli Lilly, the Federal Circuit concluded that a claim to a microorganism containing a human insulin cDNA was not adequately described by a statement that the invention included human insulin cDNA, 43 USPQ2d at 1405. The recitation of the term human insulin cDNA conveyed no distinguishing information about the identity of the claimed DNA sequence, such as its relevant structural or physical characteristics.

Eli Lilly held that an adequate written description of genetic material

> "requires a precise definition, such as by structure, formula, chemical name, or physical properties," not a mere wish or plan for obtaining the claimed chemical invention. [emphasis added]

43 USPQ2d at 1404 (quoting Fiers at 25 USPQ2d at 1606).

The disclosure must allow one skilled in the art to visualize or recognize the identity of the subject matter of the claim, 43 USPQ2d at 1406. A description of what the genetic material does, rather than of what it is, does not suffice. The holding in Eli Lilly is fatal to the sufficiency of the disclosure of the Wang Specification as filed.

Enzo Biochem, Inc. v. Gen-Probe, Inc. 62 USPQ2d 1289 (Fed. Cir. 2002), modified on Re-hearing, 63 USPQ2d 1609 (Fed. Cir. 2002), dealt with the '659 patent, which was directed to nucleic acid probes that selectively hybridize to the genetic material of the bacteria that cause gonorrhea, *Neisseria gonorrhoeae*. *N. gonorrhoeae* reportedly has between eighty and ninety-three percent homology with *Neisseria meningitidis*. '659 patent, col. 2, lines 61-64. Such a high degree of homology made detection of *N. gonorrhoeae* difficult, as any probe capable of detecting *N. gonorrhoeae* may also show a positive when only *N. meningitidis* is present. Enzo recognized the need for a chromosomal DNA probe specific for *N. gonorrhoeae*, and it derived three such probes that preferentially hybridized to six common strains of *N. gonorrhoeae* over six common strains of *N. meningitidis*. Id at col. 3, line 49 to col. 4, line 14; col. 4, lines 45-50. The inventors believed that if the preferential hybridization ratio of *N. gonorrhoeae* to *N. meningitidis* were greater than above five to one, then the "discrete nucleotide sequence will hybridize to virtually all strains of *Neisseria gonorrhoeae* and to no strain of *Neisseria meningitidis*." Id. at col. 12, lines 60-65. The three probes that the inventors actually derived had a selective hybridization ratio of greater than fifty. Id. at col. 13, lines 9-15. Enzo deposited those probes in the form of a recombinant DNA molecule within an *E. coli* bacterial host at the American Type Culture Collection. Id. at col. 13, lines 27-31.

Enzo's claim 1, in relevant part, was as follows:

1. A composition of matter that is specific for *Neisseria gonorrhoeae* comprising at least one nucleotide sequence for which the ratio of the amount of said sequence which hybridizes to chromosomal DNA of *Neisseria gonorrhoeae* to the amount of said sequence which hybridizes to the chromosomal DNA of *Neisseria meningitidis* is greater than about five, said ratio being obtained by a method comprising the folllowing [sic] steps;

Id. at col. 27, lines 29-36 (emphasis added). The method steps that follow were directed to obtaining the claimed ratio. Id. at col. 27, line 37 to col. 28, line 26. Claim 4 was directed to the deposited probes (referenced by their accession numbers) and variations thereof as follows:

4. The composition of claim 1 wherein said nucleotide sequences are selected from the group consisting of:

a. the *Neisseria gonorrheae* [sic] DNA insert of ATCC 53409, ATCC 53410 and ATCC 53411, and discrete nucleotide sequences thereof,

b. mutated discrete nucleotide sequences of any of the foregoing inserts that are within said hybridization ratio and subsequences thereof; and

c. mixtures thereof.

Id. at col. 28, lines 31-39. Claim 6 was directed to a method of conducting a hybridization assay with the deposited probes and variations thereof. Id. at col. 28, lines 47-56. The Federal Circuit held that a description of the claimed genus of nucleotide sequences by its hybridization ratio does not satisfy 35 U.S.C. 112.

The Federal Circuit concluded that the specification failed to provide an adequate written description because the claimed nucleotide sequence was described only by its binding to *N. gonorrhoeae* in a preferential ratio of "greater than about five" with respect to *N. meningitidis*. It did not describe the probe itself because the hybridization distinguished the claimed nucleotide sequences from unclaimed nucleotide sequences only by what they do, which is a purely functional distinction.

The foregoing cases, Eli Lilly specifically, hold that the original flawed sequence disclosures of Wang's sequences VIII and IX were not legally sufficient disclosures to establish entitlement to a July 26, 1990 filing date.

The cited European Patent Application No. 318216 (Houghton/Chiron) discloses only about 75% of the nucleotide sequence of HCV and represents only the nonstructural genes. There is no disclosure in Houghton of any capsid sequences.

SUMMARY

Wang's July 26, 1990 filing was fatally equivocal in its original disclosure of the HCV sequences of XIIIE and IXD, and did not actually disclose which of those critical sequences were correct until long after applicant's filing date of August 27, 1990 (and then only by presenting what would have been an arbitrary choice but for subsequently acquired information constituting new matter). Houghton is completely devoid of any disclosure of structural HCV genes including the capsid.

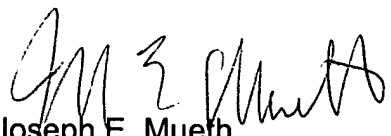
Consequently, the combination of Wang with Houghton does not result in the HCV assay methods providing early detection forming the subject matter of the present invention.

The rejection of Wang with Houghton should be withdrawn.

Reconsideration and the issuance of the Notice of Allowance is requested.

Date: January 31, 2007

Respectfully submitted,



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An Assay for Circulating Antibodies to a Major Etiologic Virus of Human Non-A, Non-B Hepatitis

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A specific assay has been developed for a blood-borne non-A, non-B hepatitis (NANBH) virus in which a polypeptide synthesized in recombinant yeast clones of the hepatitis C virus (HCV) is used to capture circulating viral antibodies. HCV antibodies were detected in six of seven human sera that were shown previously to transmit NANBH to chimpanzees. Assays of ten blood transfusions in the United States that resulted in chronic NANBH revealed that there was at least one positive blood donor in nine of these cases and that all ten recipients seroconverted during their illnesses. About 80 percent of chronic, post-transfusion NANBH (PT-NANBH) patients from Italy and Japan had circulating HCV antibody; a much lower frequency (15 percent) was observed in acute, resolving infections. In addition, 58 percent of NANBH patients from the United States with no identifiable source of parenteral exposure to the virus were also positive for HCV antibody. These data indicate that HCV is a major cause of NANBH throughout the world.

VIRAL HEPATITIS COMMONLY OCCURS in the absence of serologic markers for such known hepatotropic agents as hepatitis A virus (HAV), hepatitis B virus (HBV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) (*1–4*). Termed non-A, non-B hepatitis (NANBH), this entity represents greater than 90% of transfusion-associated hepatitis cases in the United States, and up to 10% of transfusions have been estimated to result in NANBH (*5, 6*). More recently, the frequent occurrence of NANBH in the absence of any obvious parenteral exposure has been well documented (*7–9*). Whereas acute disease is often subclinical, at least half of NANBH infections result in chronic hepatitis, which may result in cirrhosis in approximately 20% of cases (*10*). A potential association with hepatocellular carcinoma has also been proposed (*11*). Because of the frequency and severity of NANBH, there is an urgent need to develop a direct diagnostic test for the causative agent or agents. We have recently cloned the genome of a NANBH agent (*12*), designated the hepatitis C virus (HCV), and now report the development and use of a recombinant-based assay for HCV antibodies.

Three overlapping clones were isolated by means of the cDNA in HCV clone 5-1-1, which was used as a hybridization probe to the original cDNA library (*12*). These clones have one common open reading frame (ORF) extending throughout them that encodes part of a viral antigen associated with NANBH (*12*). This continuous ORF was reconstructed from these clones and then expressed in yeast (*13*) as a fusion polypeptide with human superoxide dismutase (SOD), which facilitates the efficient expression of foreign proteins in yeast and bacteria (*13–15*). In this way, a SOD/HCV polypeptide (C100-3) containing 363 viral amino acids was synthesized at high levels (~4% total protein) in recombinant yeast. After solubilization and purification, C100-3 was used to coat the wells of microtiter plates so that circulating HCV antibodies in blood samples could be captured and measured. Detection of bound antibody was achieved with a radioactive second antibody.

Initially, to test the specificity and sensitivity of this assay, sera of known NANBH infectivity was assayed in a blind fashion (Table 1). This panel of well-pedigreed and well-characterized samples has been accepted widely as a crucial test of the validity of putative specific assays for NANBH (*16*). Of seven NANBH serum samples shown to be infectious in chimpanzees, all but one gave very high signals in the assay as compared to the results obtained with sera from two control patients with alcoholic hepatitis or primary biliary cirrhosis and five non-infectious normal blood donors. These results were reproducible in quadruplicate analysis (Table 1). The only proven infectious sample that was negative in the assay was obtained from an individual in the acute phase of post-transfusion NANBH (PT-NANBH), although another acute-phase serum of unproven infectivity was similarly negative. A blood donor implicated in transmission of NANBH but whose serum was of equivocal infectivity in chimpanzees was also found negative in this assay. Thus, the data from this panel of sera indicates a high sensitivity and specificity of the antibody assay for blood-borne NANBH. No other assay evaluated by this panel has achieved this degree of specificity and sensitivity (*16*).

Next, we assayed matched blood donor and prospectively obtained recipient sera from ten well-characterized cases of chronic PT-NANBH in the United States. The results of the HCV antibody assays of sequential samples taken at 3-month intervals from each recipient during the development of NANBH and in stored samples from the corresponding donors are shown (Table 2). Each of the ten recipients seroconverted against HCV during the course of disease, although seroconversion in case 4 was marginal and not apparent until 12 months after transfusion. In contrast, seroconversion against HCV was not observed in prospectively studied individuals infected with other viral hepatitis agents. Antibody seroconversion was generally detectable within 6 months of transfusion. The prolonged interval to antibody development may explain the observed absence of HCV antibodies in the acute-phase samples assayed in Table 1.

With one exception, significant levels of HCV antibody were detected in at least one

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Table 1. Detection of HCV antibodies in proven infectious blood samples. Assays were performed (*22*) under code and in quadruplicate on a panel (*16*) containing sera from three patients with biopsy-proven chronic PT-NANBH, three implicated blood donors, and one patient with acute PT-NANBH, all of which had been proven to transmit NANBH to chimpanzees. Also included were sera from a patient with acute NANBH and a donor thrice implicated in NANBH, each of which were equivocally infectious in the chimpanzee. Control sera were assayed from five normal blood donors who had each donated blood on at least ten occasions without the development of NANBH in the recipients, from a patient with alcoholic hepatitis, and from an individual with primary biliary cirrhosis. Sera scoring positive in these assays were negative when purified SOD was used to coat wells instead of C100-3. Such samples were also positive in immunoblot analyses containing recombinant HCV polypeptides, but not SOD alone (*12*).

Serum	Counts per minute			
Proven infectious in chimp				
Chronic NANBH patients				
1 (PT-NANBH)	31,962	32,107	32,121	28,584
2 (PT-NANBH)	22,871	17,483	21,623	19,863
3 (PT-NANBH)	25,381	20,983	21,039	20,047
Acute PT-NANBH patient	909	726	767	580
Implicated blood donors				
1	40,883	33,521	35,870	34,526
2	25,812	23,512	26,476	23,723
3	31,495	30,907	33,723	33,043
Unproven infectivity in chimp				
Acute PT-NANBH patient	1,207	740	1,786	1,489
Implicated blood donor	590	469	477	461
Pedigreed normal controls				
Blood donors				
1	998	775	647	584
2	887	632	561	469
3	591	446	459	327
4	634	533	758	649
5	584	531	553	429
Disease controls				
Alcoholic hepatitis	842	571	586	566
Primary biliary cirrhosis	915	1,118	741	750

Table 2. Detection of HCV antibodies in the blood donors and recipients of ten cases of chronic PT-NANBH from the United States. There were 138 blood donations of apparent negativity that closely followed a normal distribution with a mean of 1536 cpm (range, 187 to 3097 cpm) and a standard deviation (SD) of 671 cpm. Samples >3549 cpm (mean + 3 SD) are considered positive. All prospectively studied blood recipients developed chronic NANBH as diagnosed by the persistent elevation of serum ALT levels (>6 months) in the absence of immunoglobulin M antibody to HAV, HBV surface antigen (HBsAg), antibody to HBsAg and HBcAg, and serologic markers for CMV and EBV infection. Biopsies from all ten patients confirmed the diagnosis of chronicity. Recipient sera were assayed at 3-month intervals (0 represents a sample obtained immediately before transfusion). Control samples consisted of sera from a prospective study of male homosexuals (*23*) that were assayed for up to 1 year after the onset of hepatitis as a result of infection with either HAV (18 cases), HBV (20 cases), or CMV (5 cases). None of these disease controls showed positive seroconversion to anti-HCV. The results of every positive donor unit are shown.

Case	Number of donors per transfusion	Anti-HCV assay (cpm)				
		Positive donors	Recipients (months)			
			0	3	6	12
1	18	3,910	1,870	3,220	13,120	26,780
2	18	4,590 3,800	2,530	1,170	11,400	20,750
3	13	6,140	1,800	1,850	14,990	4,720
4	18	None	1,430	1,370	750	4,260
5	16	24,420	2,230	790	13,960	22,020
6	11	6,080 25,600	2,100	10,160*	21,490	24,900
7	15	15,970	2,120	2,090	10,470	16,140
8	20	13,240	1,920	2,860	8,160	22,510
9	8†	32,790	3,370	5,800*	4,700	11,380
10	15	20,430 19,760	1,530	5,830*	19,960	20,580

*These moderately high counts per minute were shown in additional studies to be due to passive transfer of antibody from donors who had high antibody titers. †Only six of the eight donors were assayed.

donor to each of the ten recipients with NANBH (Table 2). Case number 4 had no positive donors and represented the recipient with the weakest seroconversion observed. Some of the positive donors had no surrogate markers for NANBH [elevated serum alanine aminotransferase (ALT) concentrations or the presence of antibody to the hepatitis B core antigen (HBcAg), or both (*6, 17–19*)]. The prevalence of HCV antibody in voluntary blood donors from New York with normal ALT levels (<45 international units per liter) and no antibody to HBcAg was about 0.5% (2 of 412). This frequency increased to 44% (16 of 36) in donors with both elevated ALT levels and antibody to HBcAg (*20*).

These data from characterized NANBH panels combined with previous data (*12*) indicate a specific association between HCV antibody and blood-borne NANBH. This conclusion was also supported from assays of other chronic PT-NANBH patients (Table 3). These cases differ from the NANBH cases cited in Tables 1 and 2 in that they were not prospectively monitored from the time of transfusion and, in many cases, only one serum sample was assayed. This may account for the observed lower prevalence of HCV antibody.

Table 3. HCV antibody in NANBH patients from the United States.

Transmission	Total patients	Percent positive
Blood transfusion	24	71*
No identifiable source (community-acquired)	59	58†

*Between one and three serum samples assayed from patients who had received transfusions and who were diagnosed with chronic NANBH on the basis of clinical symptoms, elevations of serum ALT for >6 months, serologic exclusion of infection with other agents (Table 2), and the exclusion of other apparent causes of liver injury. †Sequential serum samples obtained prospectively up to 3 years after the onset of clinical hepatitis associated with elevated serum ALT in the absence of serologic markers for other agents (Table 2) and other identifiable causes of liver injury.

Table 4. HCV antibody in PT-NANBH cases from Italy and Japan.

Country	Number of patients	Disease	Percent positive
Italy	32	Chronic	84*
Japan	23	Chronic	78†
Japan	13	Acute, resolving	15†

*Serum samples (about three) assayed from each patient with transfusion-related chronic NANBH (diagnosed as in Tables 2 and 3). †A prospective study in which sequential serum samples were assayed for at least 6 months after the onset of acute NANBH (diagnosed as in Tables 2 and 3). The serum ALT of acute, resolving patients returned to normal and stable levels, whereas chronic patients displayed abnormal levels for at least 6 months.

Assays were also performed on a group of patients with well-defined clinical NANBH who were prospectively monitored for up to 3 years after onset of illness but who had no identifiable source of infection (*9*). More than 50% of these individuals were either positive for HCV antibody at the time of the initial consultation with the physician or seroconverted subsequently (Table 3). Thus, it appears that HCV is a major cause of community-acquired NANBH as well as PT-NANBH.

To initiate investigations into the contribution of HCV to global NANBH, a collection of sera from NANBH patients from Italy and Japan was assayed for HCV antibody. The results indicate that 84% of Italian patients diagnosed with chronic PT-NANBH contained HCV antibody (Table 4). A similar frequency was observed in prospectively studied chronic PT-NANBH cases from Japan, but a much lower prevalence was seen in Japanese patients with NANBH that had resolved their acute infection without progression to chronic hepatitis (Table 4). The lower incidence of antibody to HCV in acute, resolving NANBH has also been observed in other human studies (*21*) and may reflect a lower stimulation of the immune system in these cases as compared with chronic, persistent infections.

These data suggest that HCV is a major cause of chronic NANBH throughout the world. The advent of the specific, sensitive test for HCV antibody described here should improve the safety of the world's blood supply as well as provide an important clinical diagnostic tool. With this assay and the availability of HCV hybridization probes (*12*), it should also be possible to address the issue of whether other parenteral NANBH agents exist.

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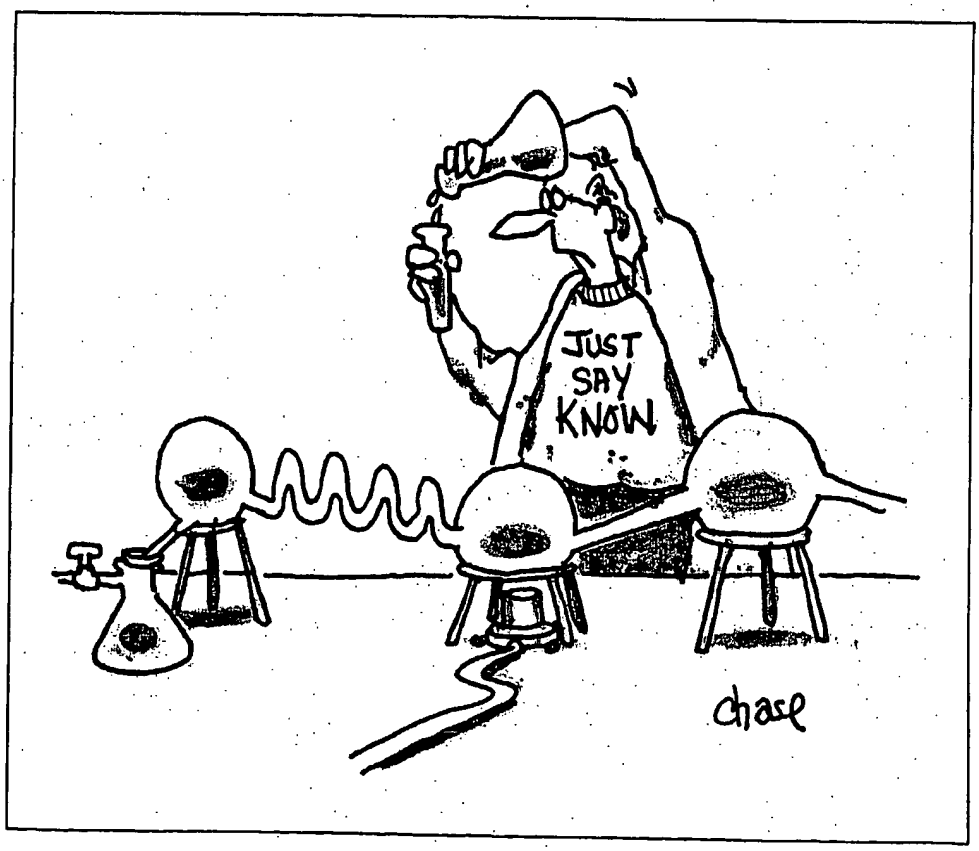

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Effects of amino acid sequence changes on antibody-antigen interactions

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Amino acid substitutions in antibody-antigen interfaces play an important role in affinity maturation of antibody responses and in antigenic variation. Structural studies show that some non-conservative changes are tolerated in these interfaces. By analogy with observations of amino acid exchangeability within homologous or mutated protein structures, this suggests that the number of different antibody specificities is less than the total number of antibodies which can be encoded by the various genetic mechanisms. On the other hand, conformational adaptability of antibody structures during binding of antigen suggests that one antibody can serve to bind a number of different antigens.

Single amino acid sequence changes within the interface of an antibody-antigen complex are important in two biological contexts. Firstly, such changes in the antibody have the capacity to drive the affinity towards more tightly bound complexes. Secondly, such changes in the antigen can effectively abolish the interaction entirely, providing an effective mechanism for antigenic variation. Although antibody and antigen display biologically asymmetric behaviour in regard to the effects of single amino acid substitutions, in principle and in practice, substitutions in either partner can raise or lower the affinity. Tolerance of amino acid sequence substitutions within antibody-antigen interfaces suggests that they

be considered examples either of protein-protein interactions generally or of the core of folded protein structures. In those cases, degeneracy of amino acid sequence information is well documented among families of homologous proteins, and it suggests that the number of different antibody specificities is less than the estimated number of different antibody molecules.

Antigenic variation

Substitutions which result in loss of binding are not amenable to direct study of complexes, although several studies have sought to rationalize the effects of the new amino acid in the context of the complex involving the "parent" amino acid.

Escape mutants of viral antigens, selected by growth of virus in the presence of monoclonal antibody, provide many examples of the type of substitution which can render the antigen unrecognizable by the selecting antibody (see, for example, Webster *et al.*, 1987). There are no discernible trends in these data which suggest preference for replacement of residues in one physico-chemical class by those in another as a preferred means of abolishing binding (Colman, 1992). Studies of the three-dimensional structures of a number of these antibody selected mutants shows that local changes, at and immediately adjacent to the site of the mutated residue, suffice to disrupt the interaction with the selecting antibody (Knossow *et al.*, 1984; Varghese *et al.*, 1988; Tulip *et al.*, 1991).

Tulip (1990) has observed that a variety of escape mutants of the influenza virus neuraminidase, selected by the anti-neuraminidase antibody NC41, result in shape changes of the antigen which could interfere with surface docking if the remainder of the antigen and the antibody were rigid and unable to relax around the mutated residue. In another case, a hydrogen bond is potentially lost by substitution to a smaller residue (Tulip, 1990). Bhat *et al.* (1990) report that substitution of Gln by His in lysozyme also causes shape changes which cannot be accommodated by the D1.3 anti-lysozyme antibody, and that, in addition, a hydrogen bond would be lost from the interaction. In both of these examples, the key to the failure of the mutant to bind may reside as much in the inflexibility of the surrounding structures as it does in the physico-chemical environment within the interface of the mutated residue.

Structural data on the effects of point changes in the binding site are available in only one system at present, the influenza virus neuraminidase (Tulip *et al.*, 1992). Two mutants of the N9 subtype neuraminidase, selected with antibodies other than NC41, involve substitutions within the binding site on N9 for NC41. In each case, the affinity is reduced by one to two orders of magnitude (Webster *et al.*, 1987; Gruen *et al.*, 1993). One of these mutants, Asn 329 to Asp, is located near the edge of the binding site for the NC41 antibody and is accommodated by re-positioning the side chain of residue 329 towards the solvent exposed perimeter of the antibody-antigen interface. The other, Ile 368 to Arg, results in a complex pattern of concerted movements around the mutation site to accommodate the arginyl residue. These changes include a shift by 3Å in the position of the arginine from its location in the uncomplexed neuraminidase structure and a shift by more than 1Å of a histidine on the antibody. In both of these structures, the effects of the mutations within the interface are reminiscent of the effects of amino acid substitutions within the interior of protein molecules (Matthews, 1991), Anderson *et al.* (1993) where localized structural rearrangements around the mutation site are frequently observed. Analysis of structures in the Brookhaven data base (Bernstein *et al.*, 1977) suggests a somewhat looser packing density of atoms within an antibody-antigen interface compared to other protein-protein interfaces or the interior of protein molecules (Tulip *et al.*, 1992; Lawrence and Colman, 1993). This suggests, in turn, that antibody-antigen interfaces should not be more sensitive to amino acid substitutions than other protein-protein interfaces.

Somatic mutation

Point mutations accumulating within the variable domains of antibody heavy and light chains are associated with increasing affinity of the antibody for antigen.

Alzari *et al.* (1990) have demonstrated that when 2-phenyloxazolone binds to an antibody, it makes direct contact with amino acids of the V_L domain which are known to be frequently mutated in antibodies with increased affinity for the antigen. In contrast, in this same system, somatic mutation in the V_H domain in no case maps to amino acids directly involved in binding the hapten.

Engineered substitutions in an anti-*p*-azophenylarsonate antibody (Sharon, 1990) have demonstrated that three of nineteen somatic changes observed in the V_H domain suffice to increase the affinity by a factor of 200. Comparison with the three-dimensional structure of the antibody (Rose *et al.*, 1990) suggests that, in every case, the effect must be indirect, since none of the three substituted residues is likely to be in direct contact with the antigen. In this example, there is not yet a report of the antibody-antigen complex structure, nor is the antibody structure highly refined, so the conclusion should be considered preliminary.

Structural context and repertoire size

The above examples paint a confusing picture of the specificity of antibody-antigen interaction. In one structural context, a very conservative substitution may abolish binding; in another, a non-conservative substitution may have very little effect on the binding affinity. How should these observations be treated if one is to estimate the effective size of the antibody repertoire from genetic sources alone? One approach is to treat the antibody-antigen interface the same way as the interior of a protein structure, and to bring to bear on this problem current knowledge about the degeneracy of "structural information" among the twenty amino acids.

Current estimates of the potential number of antibody molecules that can be generated by all the known genetic mechanisms is in excess of 10^{18} (Hunkapiller and Hood, 1989). This and similar other estimates assume each of the 20 amino acids is different from every other amino acid, which is appropriate for purposes of enumeration but not for the purpose of estimating how many different antibody specificities can be produced by an animal.

Data from exchangeability matrices (Dayhoff *et al.*, 1978) or from degeneracy of information in amino acid sequences resulting from overlapping genes (Sander and Schulz, 1979) suggest that, for structural purposes, or more precisely for folding purposes, there are effectively only four or five "different" amino acids. Can the same reduction be applied to binding interactions between proteins? Folding could be considered to be more tolerant of amino acid substitutions because of the cooperative effects caused by the interactants being covalently linked to each other. Binding interactions could be considered less tolerant because the changes involved occur in what might be called the active site. Outside of the antibody-antigen system, mapping of protein-protein interacting surfaces by mutational analysis is also generally successful (Bowie *et al.*, 1990), but some unexpected and unexplained findings do occur (de Voss *et al.*, 1992), suggesting a measure of tolerance of amino acid substitutions generally in protein-protein interfaces.

These arguments affect considerations of antibody repertoire sizes. In the facile extreme of substituting five (types of amino acids) for twenty in the calculations of Hunkapiller and Hood (1989), the expected number of different antibody specificities is reduced to order 10^8. Another way to estimate the number of different specificities is to argue from the physical size of a typical binding site on antibody for antigen. For protein antigens, this surface size is of the order of 15 amino acids. The numbers of different specificities that can be encoded over such a surface based on twenty or five structurally different amino acids are order 10^{19} and 10^{10}, respectively, if one ignores the important influences of overall surface shape and partiality of solvent exposure of amino acids within the surface. Similar arguments and estimates of repertoire size are applicable to T-cell receptors.

Some compromise in the capacity of the immune system to cope with "foreign" structures is implied by these degeneracy arguments. The above estimates have carried the argument to the extreme and could therefore be viewed as an extreme lower limit of repertoire size. Nevertheless, genetic sources of diversity are only part of the story. Antibodies, as proteins, display the usual types of conformational adaptability in binding to ligands, as do other proteins, *i.e.* side chain rearrangements and main chain changes within loop structures (Colman, 1988; Wilson and Stanfield, 1993). In addition, there is growing experimental evidence for the functioning of the V_L-V_H interface as a structural adaptor allowing movements of the heavy chain CDR *en masse* with respect to the light chain CDR during engagement with antigen (Colman, 1988, 1991; Herron *et al.*, 1991; Bhat *et al.*, 1990; Stanfield *et al.*, 1993). These changes in antibody structure are believed to be specific to the interacting antigen. Different antigens may therefore induce different structural responses in the same antibody, adding a structural dimension to diversity (Colman, 1988).

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Recognition of carbohydrates by antibodies

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The recognition by antibodies of complex polysaccharides forming the outer layer of cell walls is an integral part of the immune response to bacterial infection and invasion by non-self cells. For these reasons, the interactions of antibodies with sugars have been extensively investigated by physicochemical and immunochemical methods (Young *et al.*, 1983; Bundle, 1989; Glaudemans, 1991; Sigurskjold and Bundle, 1992). The conformation of free (Bush, 1992) and antibody-bound oligosaccharides has been studied by NMR spectroscopy (Glaudemans, 1991; Bundle *et al.*, submitted) and crystallography (Cygler *et al.*, 1991; Vyas *et al.*, 1993). NMR measurements showed that in some cases, binding to an antibody